Veterinary Microbiology 220 (2018) 97–106

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Neutral barcoding of genomes reveals the dynamics of *Salmonella* colonization in cattle and their peripheral lymph nodes

Steffen Porwollik[a], Ken Genovese[b], Weiping Chu[a], Guy H. Loneragan[c], Tom Edrington[b,1], Michael McClelland[a,*]

[a] Dept. of Microbiology and Molecular Genetics, UC Irvine, B240 Med Sci Bldg., Irvine, CA 92697, United States
[b] ARS, Food and Feed Safety Research Unit, 2881 F and B Rd, College Station, TX, 77845, United States
[c] Dept. of Animal and Food Sciences, Texas Tech University, Box 42141, Lubbock, TX, 79409, United States

ARTICLE INFO

Keywords:
Salmonella
Peripheral lymph nodes
PLN
Lifestock
Wild-type isogenic tagged strains
WITS
Sequence tag-based analysis of microbial populations
STAMP
Founding population size

ABSTRACT

Feedlot cattle often contain *Salmonella*. The number of bacteria that initiate colonization of different cattle organs and the bacterial migration within these large animals are poorly understood. To investigate these questions, we constructed **w**ild-type **i**sogenic **t**agged **s**trains (WITS) of *Salmonella* by inserting 21-base barcodes flanked by Illumina sequencing primers into a neutral genome location. We then delivered several different pools of uniquely barcoded clones orally and into multiple intradermal sites, in individual Holstein steers, and subsequently performed *Salmonella*-directed **s**equence **t**ag-based **a**nalysis of **m**icrobial **p**opulations (STAMP). Using high-throughput sequencing of the barcodes of *Salmonella* grown from steer lymph nodes, organs and feces, we monitored how individual barcoded clones travel from different entry sites within animals. Data showed that gastrointestinal colonization was established by up to hundreds of *Salmonella* founder cells, whereas peripheral lymph nodes were usually colonized by very low numbers of founding bacteria, often originating from the nearest draining intradermal delivery site. Transmission of *Salmonella* from the gastrointestinal tract to the lymphatic system was frequently observed, whereas entry of intradermally delivered bacteria into the gut was rare. Bacteria undergo limited extraintestinal proliferation within or prior to arrival at peripheral lymph nodes. Overall, the application of the STAMP technique facilitated characterization of the migration routes and founder population size of *Salmonella* within feedlot cattle and their organs and lymph nodes in unprecedented detail.

1. Introduction

Salmonella enterica is often found in the feces of cattle, at frequencies that vary depending on geographical location, season, and detection method (Brashears and Chaves, 2017; Kunze et al., 2008; Loneragan and Brashears, 2005), but can reach over 90% of animals in a given feedlot (Narvaez-Bravo et al., 2013). A nation-wide USDA study estimated presence of the bacterium in over 60% of US cattle feedlots (http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_is_Salm.pdf).

Most concerning is the presence of the bacterium in peripheral lymph nodes (PLNs) of healthy cattle presented for harvest (Arthur et al., 2008; Brichta-Harhay et al., 2012; Gragg et al., 2013a; Gragg et al., 2013b; Haneklaus et al., 2012; Koohmaraie et al., 2012; Webb et al., 2017). PLNs are a potential source of human exposure to *Salmonella*, particularly when adipose trim containing these nodes is incorporated into ground beef. The median *Salmonella* prevalence in thousands of subiliac lymph nodes of feedlot cattle from seven US-based plants was 11.8%, and of the *S. enterica*-positive animals, 42% contained more than 1000 colony-forming units (CFUs) per lymph node (Gragg et al., 2013a). A more recent study estimated the presence of *Salmonella* in PLNs of feedlot-fattened and cull cattle to average at around 5.3%, with notable regional and seasonal differences (Webb et al., 2017). *Salmonella* inside lymph nodes are protected from routine carcass interventions. Therefore, pre-harvest interventions are needed to minimize the presence of *Salmonella* in ground beef product.

Testing new interventions requires a reliable model for PLN colonization. Oral challenge doses as high as 10^{10} CFUs did not always produce successful colonization of PLNs (Edrington et al., 2013b). However, experimental inoculation of horn flies with *Salmonella* and subsequent exposure of cattle to these flies resulted in transmission of *Salmonella* to the PLNs (Olafson et al., 2016). Similarly, intradermal

* Corresponding author.
E-mail addresses: sporwoll@uci.edu (S. Porwollik), genovese@sparc.usda.gov (K. Genovese), weipingc@uci.edu (W. Chu), guy.loneragan@ttu.edu (G.H. Loneragan), tedrington@diamondv.com (T. Edrington), mmcclell@uci.edu (M. McClelland).
[1] Present address: Diamond V Mills, Inc., Cedar Rapids, IA United States.

https://doi.org/10.1016/j.vetmic.2018.05.007
Received 4 April 2018; Received in revised form 15 May 2018; Accepted 15 May 2018

delivery leads to frequent colonization of nearby PLNs (Edrington et al., 2016; Edrington et al., 2013a; Edrington et al., 2013b). The intradermal delivery method worked in steers of widely different sizes and predictably resulted in *Salmonella*-positive PLNs for at least 28 days post inoculation.

To date, it remains unknown how *Salmonella* travels within steers to reach PLNs or various organs. Recent innovations in molecular barcoding techniques and sequencing technology fostered the development of a novel strategy, called **s**equence **t**ag-based **a**nalysis of **m**icrobial **p**opulations (STAMP) (Abel et al., 2015). This strategy was established as a way to determine biological population bottlenecks, and builds on the concept of **w**ild-type **i**sogenic **t**agged **s**trains (WITS) (Grant et al., 2008), where identifying sequences are placed in a neutral location in a bacterial genome and are either PCR amplified or sequenced from bacterial populations before and after exposure to a biological environment. WITS has been applied to reveal intra-animal migration patterns of *Salmonella* to murine cecal lymph nodes (Kaiser et al., 2013), to characterize *Salmonella* migration between murine systemic organs and gut lymphoid tissue (Lim et al., 2014), and to investigate *Salmonella* inter-animal transmission routes in chicken (Yang et al., 2017). STAMP expands WITS by multiplying the number of tagged pathogens used and considering relative abundances of each tagged isolate, so that more complex founding populations can be determined (Abel et al., 2015). Importantly, this advancement minimizes the number of biological experiments (i.e. animals) that are needed for robust estimations of population sizes and migration patterns.

In this report, we utilized both oral and intradermal delivery to study the dynamics of bacterial growth, dissemination and survival in cattle feces, organs and PLNs, at a resolution beyond the total bacterial load. We barcoded the genomes of 85 clones from eight *Salmonella* strains that had been isolated from PLNs of feedlot cattle and delivered multiple non-overlapping sets of barcoded clones to many different locations into steers, at different times and doses. We subsequently identified the delivery route that led to colonization of particular organs or lymph nodes, and studied *Salmonella* dissemination and replication in those steers, with particular focus on the animals' PLNs.

2. Methods

2.1. Animal upkeep

Holstein steers were purchased from a single supplier. Upon arrival, steers were administered tulathromycin (Draxxin®, Pfizer Animal Health, New York City, NY) and a broad-spectrum bacterial/viral vaccine (ViraShield® 6, Novartis Animal Health, Greensboro, NC), and wormed (Dectomax, Pfizer Animal Health, New York City, NY). Subsequently, steers were maintained on grass pasture. A commercial beef cattle feed was fed at approximately 2.3 kg/head/day until the steer was assigned to experiments.

Prior to the experiment, feces were collected from all animals on four occasions and pre-screened for the presence of *Salmonella*. Animals were halter trained for five days prior to moving to the indoor livestock facility. Unless indicated otherwise, the steers were housed individually in concrete-floored pens (11 × 11 feet) with water available *ad libitum*. Cattle were fed a 50:50 mix of a non-medicated commercial cattle feed and grass hay at 2.5% of their bodyweight twice daily.

2.2. Salmonella inoculation of steers

Bacteria were delivered to steers by the oral route or intradermally at eight different sites on the animal (Fig. 1), in two different inoculation schemes (Fig. 2, see below for details). Animals were inoculated with clones derived from multiple naturally occurring *Salmonella* strains obtained from feedlot cattle (Table 1). These clones were strategically pooled, as described in Table 2. The most complex pools were administered orally by nasogastric tube (25 ml). Following administration of the inoculum, 100 ml of sterile water was administered to ensure that the entire inoculum was delivered to the rumen. Additional *S. enterica* mutant pools were administered intradermally, using previously published methods (Edrington et al., 2013b). Briefly, a commercially available multi-lancet device (ComforTen® Multiple Skin Test Device, Hollister-Steir Allergy) was used, consisting of 10 testing probes fitted with a lancet tip that provides intradermal, but not subcutaneous, administration of the *Salmonella*. The device was dipped into Luria broth containing different *Salmonella* pools and then applied to each location. Four applications of the device were made to each inoculation site. The device was dipped into the *Salmonella* broth prior to each administration and a new device was utilized for each of the different *Salmonella* pools. Overall concentrations of the bacterial pools ranged from 2.3×10^8 to 10.6×10^8 CFU/ml and averaged 5.8×10^8 CFU/ml. Note that representation of individual clones in these pools varied, as described in Table 2.

Fecal samples were collected via rectal palpation daily throughout each experiment. At the end of the experiment, steers were euthanized (Euthasol®, euthanasia solution; Delmarva Laboratories, Inc., Midlothian VA) and necropsied. Subiliac (aka pre-femoral, FEM), popliteal (POP), and superficial cervical (aka pre-scapular, SCAP) lymph nodes (right and left) were collected and cultured for *Salmonella* as described below. Mesenteric lymph nodes (MES), as well as tissues from the liver, lung and spleen were also collected. The luminal contents from the cecum and spiral colon were collected last to prevent contamination of the tissue and lymph node samples. All experiments involving live animals were approved by the Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA.

2.3. Tissue processing and bacterial culture

Lymph nodes, lung, spleen and liver tissue samples were trimmed of excess fat and fascia, and surface sterilized by immersion in boiling water for 3 s. The sterilized organ was placed into a filtered stomacher bag and the tissue pulverized using a rubber mallet. Tetrathionate broth (80 ml) was added to each sample bag followed by mixing for 30 s with a laboratory blender. Quantification and qualification of the bacterial strains was conducted as described previously (Edrington et al., 2013a; Edrington et al., 2013b). For quantitative measurements, 1 ml of the pulverized tissue/tetrathionate broth mixture was removed and spread on agar selective for Enterobacteriaceae (Petrifilm™ EB, 3 M Health Care, St. Paul, MN) in duplicate and incubated (overnight, 37 °C). Films with bacterial growth were used to surface-inoculate XLD plates (containing 10 μg/ml cefsoludin and 15 μg/ml novobiocin) and incubated (37 °C, 24 h). Black colonies were counted and converted to CFU/tissue. For qualitative detection, the tissue/tetrathionate broth mixture was kept at room temperature for 2 h, incubated overnight (37 °C), then 100 μl were transferred to 3 ml Rappaport-Vassiliadis broth (RV; Remel Products, Lenexa, KS) and incubated at 42 °C for 24 h. This enrichment was subsequently plated on brilliant green agar supplemented with sulphadiazanine (80 μg/ml; BGAs). Plates were incubated (37 °C, overnight) and *Salmonella* positive plates recorded.

Fecal samples and luminal content samples from the cecum and spiral colon were cultured as follows: 10 g of each sample were mixed with 90 ml of tetrathionate broth. Exactly 1 ml of this mixture was removed and 50 μl were plated on xylose lysine deoxycholate agar [XLD, Oxoid, Basingstoke, Hampshire, UK; supplemented with novobiocin and naladixic acid (25 and 20 μg/ml, respectively)] using a spiral plater (Spiral Biotech Autoplate 4000, Advanced Instruments, Inc., Norwood, MA). Plates were incubated overnight (37 °C) and black colonies counted and converted to CFUs. The tetrathionate/sample mixture was incubated overnight at 37 °C prior to a sub-sample (100 μl) being transferred to 5 ml of RV broth and incubated at 42 °C for 24 h. The RV enrichment was plated on brilliant green agar (Oxoid Ltd, Basingstoke, Hampshire, UK) supplemented with novobiocin and naladixic acid (25 and 20 μg/ml, respectively) and plates incubated (37 °C, overnight).

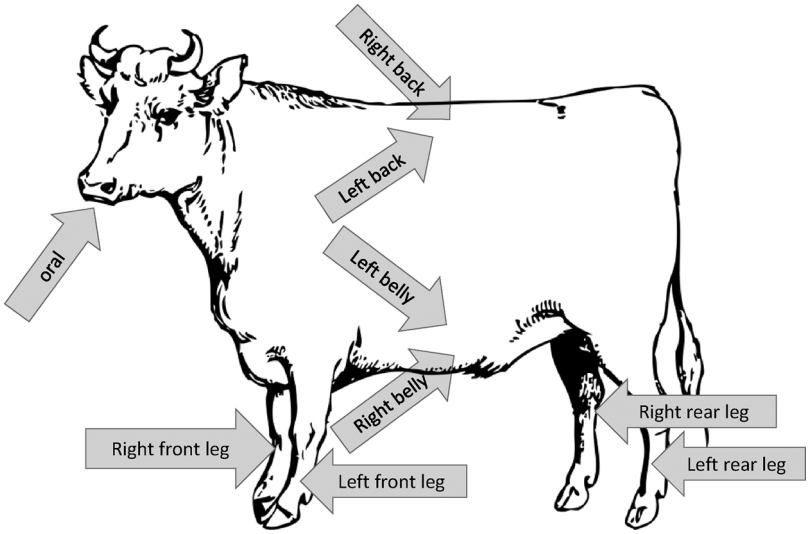

Fig. 1. Oral delivery and intradermal injection sites of barcoded *Salmonella enterica* into Holstein steers.

In all cases, 1 ml of the tetrathionate mixtures and of the RV enrichment mixtures of tissue, feces or luminal contents were supplemented with glycerol (20%) and frozen for later DNA analysis.

2.4. Experimental design

The overall design of experiments is illustrated in Fig. 2. In inoculation *scheme S*, barcoded clones were administered orally or intradermally only once. Eight individually housed Holstein steers were inoculated on day 0 with *Salmonella* as described above using pools TE1 – TE9 (Table 2). Two animals (S1 and S2) were euthanized three days post inoculation, and six additional animals were euthanized at seven days post inoculation (S3 – S8). Seven of the animals were actively shedding *Salmonella* in their feces prior to experimental inoculation.

In inoculation *scheme M*, barcoded clones were administered orally only once (day 0), but intradermally on five consecutive days. Four Holstein steers were used. Two steers were inoculated with *Salmonella* A

Table 1
Salmonella enterica strains previously isolated from cattle peripheral lymph nodes and barcoded for this study. * Strain numbers are from the Texas Tech University strain collection.

Strain Abbreviation	*Salmonella enterica* Serovar	Strain Number*	Alternate Name	Number of Barcoded Clones Obtained
SMO-1	Montevideo	12TTU1271X	MZ2269	24
SMO-2	Montevideo	11TTU382B	MZ2274	10
SMO-3	Montevideo	11TTU1694B	MZ2284	10
SAN-4	Anatum	11TTU577B	MZ2275	11
SMG-5	Meleagridis	12TTU1464B	MZ2280	10
SMG-6	Meleagridis	11TTU535B	MZ2283	9
SKY-7	Kentucky	12TTU1928X	MZ2272	10
SKY-8	Kentucky	11TTU1854B	MZ2278	1

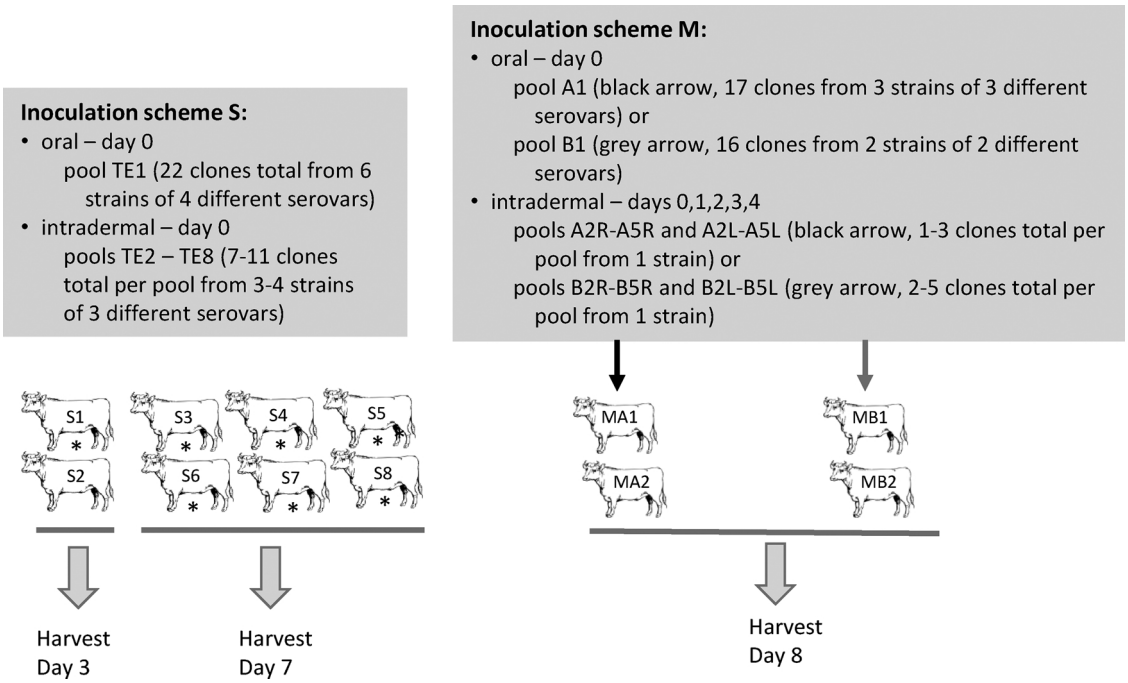

Fig. 2. Experimental design. Note that all animals received oral and intradermal inoculations. *Cow with pre-existing native *Salmonella*.

Table 2
Pools of barcoded neutral *S. enterica* mutants used in this study and their administration route. SMO, *S. enterica* serovar Montevideo; SAN, *S. enterica* sv Anatum; SMG, *S. enterica* sv Meleagridis; SKY, *S. enterica* sv Kentucky.

Pool Name [R = right, L = left]	Inoculation Scheme	Pool Complexity [No of Clones]	Inoculation Site	Clones [strain (# of barcoded clones)]	Serial Dilution Factor for Multiple Clones of the Same Strain
TE1	S	22	Oral	SMO-1 (5); SMO-2 (5); SMO-3 (1); SAN-4 (5); SMG-5 (1); SKY-7 (5)	4
TE2	S	7	Right front leg	SMO-1 (5); SMG-6 (1); SKY-8 (1)	4
TE3	S	7	Right rear leg	SMO-2 (5); SMG-5 (1); SMG-6 (1)	4
TE4	S	7	Right belly	SMO-3 (5); SMG-5 (1); SMG-6 (1)	4
TE5	S	11	Left back	SMO-1 (5); SAN-4 (5); SMG-6 (1)	4
TE6	S	7	Left front leg	SMO-1 (1); SMG-5 (5); SMG-6 (1)	4
TE7	S	7	Left rear leg	SMO-1 (1); SMO-3 (1); SMG-6 (5)	4
TE8	S	8	Left belly	SMO-1 (1); SMO-3 (1); SMG-5 (1); SKY-7 (5)	4
TE9	S	7	Right back	SMO-1 (5); SMO-3 (1); SMG-5 (1)	4
A1	M	17	Oral	SMO-1 (6); SMO-2 (10); SKY-8 (1)	4 (SMO-1), 2 (SMO-2)
A2R	M	2	Right front leg	SKY-7 (2)	1
A3R	M	3	Right rear leg	SMO-1 (3)	1
A4R	M	2	Right belly	SMG-6 (2)	1
A5R	M	1	Right back	SMG-6 (1)	n/a
A2L	M	2	Left front leg	SKY-7 (2)	1
A3L / A4L	M	3	Left rear leg / belly	SMO-1 (3)	1
A5L	M	1	Left back	SMG-6 (1)	n/a
B1	M	16	Oral	SMO-1 (6); SMG-5 (10)	4 (SMO-1), 2 (SMG-5)
B2R	M	5	Right front leg	SMO-3 (5)	1
B3R	M	2	Right rear leg	SAN-4 (2)	1
B4R	M	3	Right belly	SMO-1 (3)	1
B5R	M	3	Right back	SAN-4 (3)	1
B2L	M	5	Left front leg	SMO-3 (5)	1
B3L	M	3	Left rear leg	SMO-1 (3)	1
B4L	M	3	Left belly	SAN-4 (3)	1
B5L	M	3	Left back	SAN-4 (3)	1

pools (Table 2), the remaining two were inoculated with B pools. On day 7, the steers were euthanized and necropsied.

2.5. Construction of clones with clone-specific bacterial neutral barcodes

The construction of barcoded *phoN*$^{-}$ *Salmonella* strains is illustrated in Fig. 3. A kanamycin resistance cassette with a different 21 base DNA barcode for each cassette molecule was generated by standard PCR in a two-step procedure. In the first PCR, unique barcodes as well as Illumina Read1 and Read2 sequences were introduced via specific primers pCLF_FP and pCLF_RP (all primer sequences are shown in Table S1), that amplified the kanamycin resistance gene from plasmid pCLF4 (GenBank: EU629214.1). A 30-cycle PCR reaction was performed in a 1 x DreamTaq reaction mix (Thermo Fisher), where annealing and extension temperature were both 75 °C. In the second PCR, DNA segments of the Salmonella *phoN* gene were appended via primers phoN_FP and phoN_RP. To perform this PCR, the product of the first PCR was gel purified in low melting point agarose. Purified PCR products were used as templates for a 40-cycle PCR reaction using 1 x DreamTaq mix, an annealing temperature of 50 °C and an extension temperature of 72 °C. The resulting PCR product was gel purified using QIAEX® II (Qiagen), and used to replace the *phoN* gene with a barcoded kanamycin resistance cassette using Lambda-Red recombination (Datsenko and Wanner, 2000). Thus, most of the *phoN* gene was replaced with a uniquely barcoded molecule of the resistance cassette.

2.6. PCR amplification of barcodes and sequencing

Amplification of the barcodes present in bacterial clones of input pools and in clones harvested from the animals was performed in a nested PCR regimen (illustrated in Fig. 4). Aliquots of approximately 5×10^7 CFU were washed and digested with proteinase K as described (de Moraes et al., 2017). Subsequently, primers pCLF_FP2 and pCLF_RP2 (Table S1) were used to amplify the right flank of the transposon insertion in a 20-cycle PCR in 25 u l of 1 x Kapa HiFi reaction mixture. One microliter of the PCR product was used as the template in a second PCR amplification for 15 cycles, using 1.25 U of Taq polymerase (Invitrogen) and mass-limiting amounts of 0.1 mM nucleotide triphosphates (dNTPs), with primers Read1_Index_N_8 and Read2_Index_N_8 (Table S1). These primers each contain a unique 8-mer index used in Illumina sequencing to distinguish among samples. Products of the second PCR were pooled and subjected to QIAquick PCR product purification (Qiagen), according to the manufacturer's recommendation. Samples were sequenced using Illumina sequencing and standard sequencing primers for a dual indexed run, with a read length of at least 21 bases.

2.7. Sequence analysis

For the identification of barcoded mutants, the first 21 bases of each sequence read represented the unique barcode for each mutant clone. These barcodes were extracted and enumerated using custom Python scripts. Prior sequencing of input pools and the ordered libraries of barcoded clones for each of the eight strains (four serovars) provided a framework of expected barcodes that was used to identify each clone. In essence, 85 21-mer tags each represented a known clone of a known strain that was then used in specifically designed pools to challenge the animals in distinctive delivery loci.

3. Results

3.1. Neutral barcoding of genomes

A library of mutants containing 21-base neutral barcodes was constructed in eight *Salmonella enterica* strains. These eight strains included three strains of serovar Montevideo (SMO-1, SMO-2 and SMO-3), one strain of Anatum (SAN-4), two strains of Meleagridis (SMG-5 and SMG-6), and two strains of Kentucky (SKY-7 and SKY-8), all of which had previously been isolated from the PLNs of cattle at harvest (Gragg et al., 2013a; Webb et al., 2017) (Table 1). Barcode tags were introduced in

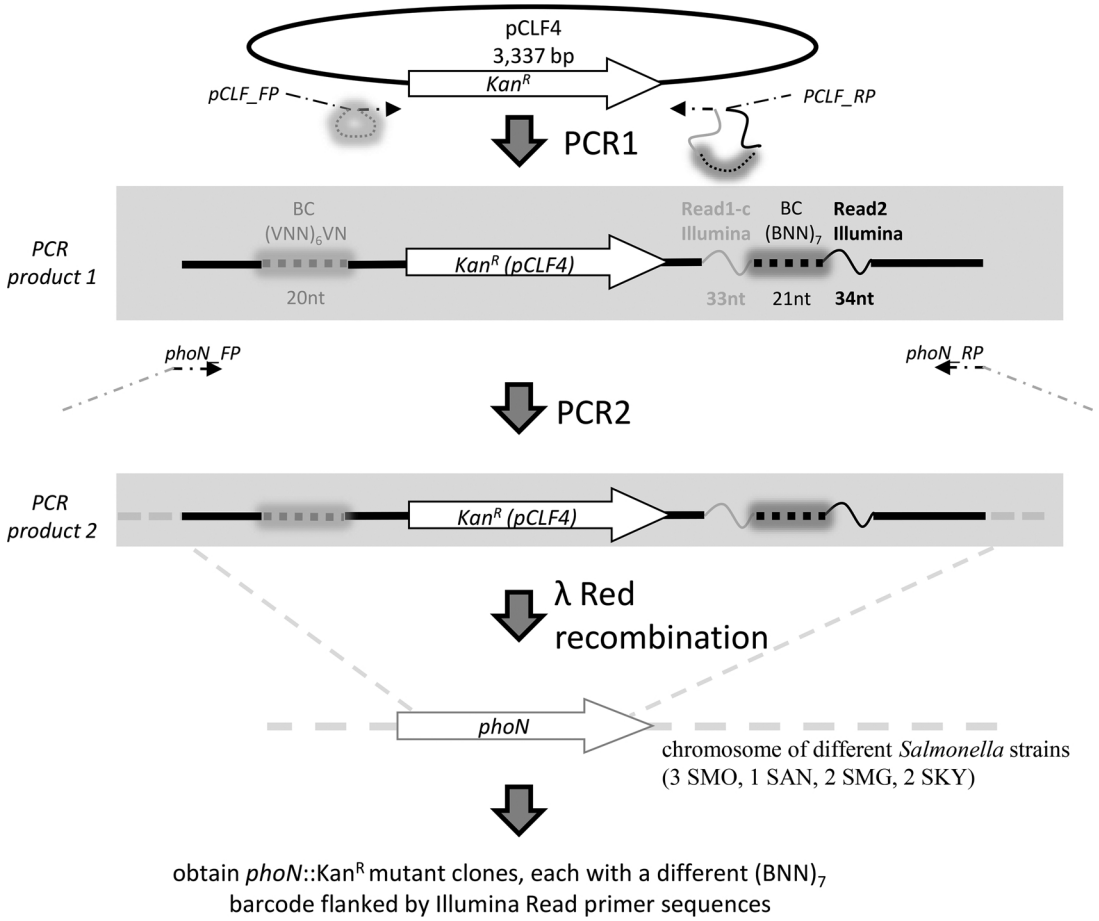

Fig. 3. Neutral barcoding of genomes for construction of kanamycin-resistant barcoded *Salmonella enterica* clones. A step-wise PCR regimen was applied, followed by Lambda Red recombination. SMO, *S. enterica* serovar Montevideo; SAN, *S. enterica* sv Anatum; SMG, *S. enterica* sv Meleagridis; SKY, *S. enterica* sv Kentucky. Overall, 85 individual barcoded clones were obtained. See Table S1 for primer sequences.

the *phoN* gene of each strain, which is a neutral location in over 1000 *in vitro* growth conditions and during colonization of a variety of mammals by *Salmonella*, including cows (Canals et al., 2012; Hao et al., 2012; Kaniuk et al., 2011; Reynolds et al., 2011; Santiviago et al., 2010; Santiviago et al., 2009; Silva et al., 2012; Weatherspoon-Griffin et al., 2011). Each barcoded clone is unique because it is randomly derived from an *in vitro* pool of over 500 billion possible different 21mer nucleotide tags. Subsequently, kanamycin resistant clones were picked into an ordered library, which included 44 Montevideo clones, 19 Meleagridis clones, 11 Anatum clones, and 11 Kentucky clones. Next, these uniquely barcoded clones were used to generate pools as described in Table 2, with each pool containing unique non-overlapping tags not found in any other pool.

3.2. Global observations

We inoculated 12 Holstein steers in two distinct inoculation schemes (Fig. 2), using a number of non-overlapping pools of barcoded *S. enterica* clones. The most complex pools of up to 22 different clones were delivered orally whereas lower complexity pools were given intradermally in eight different locations (Fig. 1). All sequence counts for each barcoded bacterium present in any of the bacterial pools used, from all animal locations where bacterial presence was detected by PCR, are shown in Table S2. This table also shows which exact barcoded clones were included in each inoculation pool.

Table 3 illustrates the overall success of bacterial barcoded clones to colonize various locations in the animal body. Table S2 confirms that at least one barcoded clone from each of the eight strains used in our experiments successfully colonized an organ, a lymph node and the intestine in at least one instance, proving that the strains' ability to colonize the animal had been retained after laboratory mutagenesis experiments.

Gastrointestinal colonization, evidenced by presence of barcoded *Salmonella* clones in the feces, was swiftly established – multiple clones were already present in the feces by the first sampling on day 1 or day 2. Notably, colonization persisted to the same extent over the entire time period investigated, and was not noticeably increased by the multiple intradermal delivery protocol in inoculation *scheme M*. However, the number of intradermal clones present in fecal matter was elevated in steer MA2. In this animal, two intradermal clones were detected in each fecal sample, in addition to multiple oral clones. In all other animals, 87.5% of fecal samples were completely devoid of any intradermal *Salmonella* clones (Table S2).

3.3. Effect of pre-existing Salmonella on colonization patterns

As illustrated in Fig. 2, seven of the twelve animals used in our study had been colonized with naturally occurring *Salmonella* clones prior to the start of the experiment. Four of these pre-existing clones had been determined by serotyping to be naturally occurring (i.e. not barcoded) serovar Montevideo isolates, while three of these had been serotyped as *rough O:g,m,s:-*, i.e. displaying identical H antigen structures compared to Montevideo strains, but lacking the O antigen. The rate of colonization of any steer location with our barcoded clones may have been affected by the presence of these pre-existing *Salmonella* in these animals. Four of the five naïve steer were utilized in inoculation *scheme M*,

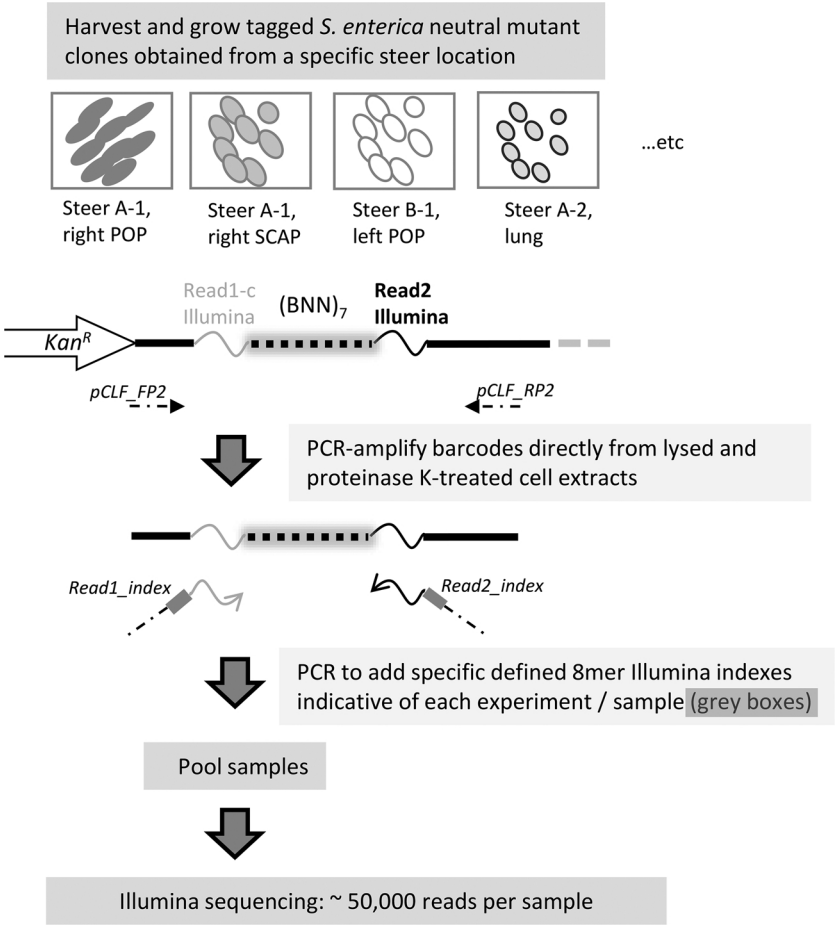

Fig. 4. Identification of barcodes of tagged *S. enterica* neutral mutants obtained from steer locations by Illumina sequencing. Up to 900 separate steer/location combinations (30 × 30 8-mer barcodes) can be interrogated in one sequencing lane. See text for experimental details, and Table S1 for primer sequences.

and therefore had no suitable counterpart to investigate the effect of the pre-existing bacterial presence. However, colonization rates and patterns of pre-exposed steer S1 were compared with those observed in naïve steer S2. Overall, the frequency of clones detected in the gastrointestinal tract (39 vs 31), the lymph nodes (15 vs 19) and the organs (14 vs 10) of each animal were comparable between steers S1 and S2.

However, we observed an inability of orally delivered barcoded *S. enterica* sv Montevideo clones to colonize PLNs in the animals subjected to inoculation *scheme S* (Table 4**).** Oral pool TE1 included 11 clones from three strains of Montevideo, the most frequent one of which represented 18% of the pool. None of these Montevideo clones were detected in any PLN of animals S1 – S8. A similar Meleagridis clone, representing 15% of TE1, successfully colonized 11 PLNs. Moreover, rarer Kentucky and Anatum clones were also found in a few PLNs of steers S1 – S8. The overall success of Montevideo clones to colonize the gastrointestinal tract in these animals was somewhat lower, compared to the clones derived from other serovars. No such effect was measurable for intradermally delivered Montevideo clones in all twelve animals, nor

Table 3
Recovery of *S. enterica* isolates from sampled bovine locations. The table lists estimated *Salmonella* CFUs per organ or node. POP, popliteal lymph node; SCAP, scapular lymph node; FEM, femoral lymph node; MES, mesenteric lymph node; L, left; R, right.

Experimental Conditions	Animal #	Location												
		FEML	FEMR	POPL	POPR	SCAPL	SCAPR	Liver	Lung	Spleen	MES	Cecum	Spiral Colon	Feces
inoculation *scheme S*: single inoculation; 3 days	S1	237	194	39	39	42	72	262	20	12	721	177828	275423	+
	S2	40	423	42	22	37	43	27	16	660	123	603	10	+
inoculation *scheme S*: single inoculation; 7 days	S3	44	39	39	38	0	0	39	38	43	2522	467735	575440	+
	S4	38	0	0	42	0	0	0	45	0	325	1202	4365	+
	S5	0	0	0	0	0	40	0	76	0	38	3162	6607	+
	S6	128	41	44	43	47	40	42	0	47	2601	102329	380189	+
	S7	113	41	0	42	57	54	39	41	0	40	977	1202	+
	S8	38	0	0	0	49	52	0	39	0	48	12882	11220	+
inoculation *scheme M*: single oral & multiple intradermal inoculation; 8 days	MA1	76	286	40	40	36	40	38	41	37	434	630957	794328	+
	MA2	741	960	71	37	38	36	38	42	41	118	630957	398107	+
	MB1	417	1520	123	133	996	985	59	36	39	2082	794328	158489	+
	MB2	40	512	393	164	72	207	40	40	36	37	1258925	794328	+

Table 4
Success of individual barcoded *Salmonella* clones present in oral pool TE1 to infect Holstein steers S1 - S8. Despite abundant presence of Montevideo clones in oral input pool TE1, none of these colonized any peripheral lymph node (PLN). SMO, *S. enterica* serovar Montevideo; SAN, *S. enterica* sv Anatum; SMG, *S.enterica* sv Meleagridis; SKY, *S. enterica* sv Kentucky.* includes fecal samples, cecum, spiral colon and mesenteric lymph node; ** includes popliteal lymph node, scapular lymph node and femoral lymph node samples; *** includes liver, lung and spleen samples.

Clone ID	Strain	Representation in Input Pool TE1 (calculated from the frequency of individual barcodes in input samples)	Number of Clone-positive Gastrointestinal Samples*	Number of Clone-positive PLNs**	Number of Clone-Positive Organs***
Serovar Kentucky					
A75	SKY-7	43.32	69	19	12
A76	SKY-7	8.12	54	1	2
A77	SKY-7	2.05	36	2	1
A78	SKY-7	0.20	20	0	0
A79	SKY-7	0.03	1	0	0
Serovar Meleagridis					
B34	SMG-5	15.24	68	11	12
Serovar Anatum					
B69	SAN-4	1.22	47	1	4
B70	SAN-4	0.33	33	1	3
B65	SAN-4	0.04	3	0	0
B64	SAN-4	0.01	3	0	0
B63	SAN-4	0.01	11	0	0
Serovar Montevideo					
B68	SMO-1	18.06	44	0	4
B67	SMO-1	5.48	41	0	4
B59	SMO-1	1.67	32	0	1
B60	SMO-1	0.31	12	0	1
B58	SMO-1	0.09	1	0	0
A13	SMO-2	2.21	14	0	1
A14	SMO-2	0.46	6	0	0
A15	SMO-2	0.14	2	0	0
A17	SMO-2	0.03	0	0	0
A16	SMO-2	0.01	0	0	0
B57	SMO-3	0.96	8	0	0

for orally delivered Montevideo clones in the four naïve steers in inoculation *scheme M*, which colonized PLNs in similar rates compared to clones from the other serovars (Table S2).

3.4. Founder population sizes of colonizing Salmonella in the gastrointestinal environment

The gastrointestinal tracts of the steer were reliably colonized with barcoded clones from the oral pools. In inoculation *scheme S* (applied to eight animals), a pool of twenty-two barcoded clones (TE1), encompassing six strains from four different *Salmonella* serovars, was orally delivered. Seven of these twenty-two clones were present (defined as > 200 barcode counts after sequencing) in at least half of all fecal, cecal and colon samples. The top three clones of strains SKY-7 and SMO-1 were often present in these samples. Since strain-specific clones had been added to TE1 in form of a 4-fold dilution series (Table 2), the recurrent representation of the three most frequent clones per strain in gastrointestinal samples was a strong indicator for a consistent founding population size of greater than 100. Clone B70, an Anatum SAN-4 clone, represents only 0.3% of TE1, and successfully colonized the cecum in six of the eight animals (Table S2). At 2.2% representation in TE1, clone A13, a Montevideo SMO-2 clone, was the most frequent TE1 clone that failed to colonize the cecum in the majority of these animals.

In inoculation *scheme M* (applied to four animals), the seventeen clones in pool A1 were orally delivered to two animals, and the sixteen clones in pool B1 to the other two. The top three barcoded SMO-1 clones in pool A1 and the top five barcoded SMG-5 clones in pool B1 were detected in the majority of the gastrointestinal samples of the animals. SMO-1 clone A04, representing 1.67% of pool A1, colonized the cecum of both animals which had ingested this pool, while pool B1 clone B34, an SMG-5 clone, did the same while representing 5.7% of that pool (Table S2). Conversely, the most abundant barcoded clone in pool A1 that failed to colonize the cecum of either of the two animals was clone A10, an SMO-2 clone, at 3.9% representation, whereas for pool B1 the most abundant "failure" was clone B43, an SMO-1 clone, at 8.5% representation. Overall, these numbers suggest the founding bacterial population size for colonization of the gut to be in the hundreds.

3.5. Founder population sizes of colonizing Salmonella in cattle organs and PLNs

Internal organs were overall less reliably colonized following experimental inoculation of the steer. In the 12 animals, colonization of livers, lungs or spleens by more than one clone occurred in 17 / 36 organs (47%). In the organs that were colonized, the colonizing clone(s) stemmed from an oral pool in over 70% of cases. Occasionally, barcoded clones from intradermal inoculations (in most cases from the rear legs) were also encountered, notably in liver and spleen samples. No serovar- or clone preference for any of the organs was observed. The highly variable number of colonizing clones and the low number of organs with more than one clone precluded a reliable and statistically significant estimate of a founder population size for internal organs of the Holstein steer.

We attempted to discern the founder population size of *Salmonella* in cattle PLNs. In inoculation *scheme S*, site-specific pools that contained between seven and eleven clones at variable representation were intradermally delivered on day zero in various locations on the animals' bodies. Subsequently, 22 of all 48 PLNs of the animals (46%) were colonized with three or more different barcoded clones, despite overall low CFU counts for each organ (Table 3), and 75% of all PLNs experienced colonization by more than one clone. However, detection of the two most frequent barcoded clones per strain in only 6 of 48 PLN samples (12.5%) suggested a low founding population size in the tens. In inoculation *scheme M*, the intradermal pools contained multiple barcoded clones of the same strain in approximately equal numbers (and not as a dilution series), and the pools were delivered daily over the course of the experiment. In this setup, 16/24 PLNs (67%) were colonized with three or more clones, and 87.5% of all PLNs by more than one clone. In 12 PLNs (50%), more than one barcoded clone of the same strain present in a specific intradermal pool was detected. A maximum of ten different barcoded clones was identified in one peripheral lymph node (FEML of animal MB1). Similar to the observations made in inoculation *scheme S*, these data suggest a typical founder population size of up to tens of these microorganisms per steer PLN in *scheme M*.

3.6. Lymphatic paths of intradermally delivered Salmonella clones

Each lymph node collects draining lymph from specific region. Therefore, bacteria inoculated into the front legs were expected to be found primarily in the superficial cervical / pre-scapular (SCAP) lymph nodes, whereas barcoded clones injected into the rear legs were expected to appear in the popliteal (POP) lymph nodes. The subiliac / prefemoral (FEM) lymph node collects draining lymph from the belly and back of the animal. Table 5 depicts the locations where clones that appeared in the different lymph nodes of the 12 animals had been introduced into the animal.

As expected, the SCAP lymph nodes primarily contained barcoded clones that were inoculated intradermally in the region drained by

Table 5
Origin of *S. enterica* clones detected in lymph nodes of the 12 steers used in this study. The total sum of clones detected in 12 cattle lymph nodes is depicted, separated by the clones' inoculation site. Note that the table depicts frequency of origin-specific clone occurrence, not number of different clones. POP, popliteal lymph node; SCAP, scapular lymph node; FEM, femoral lymph node; MES, mesenteric lymph node; L, left; R, right. The top numbers in each row and column are in bold.

	Harvest Site						
Inoculation Site	FEML	FEMR	POPL	POPR	SCAPL	SCAPR	MES
Oral	**15**	9	2	6	7	6	**44**
Left front leg	0	0	0	3	**9**	0	0
Right front leg	1	0	0	0	0	**15**	0
Left rear leg	**11**	1	**10**	0	0	1	0
Right rear leg	1	**10**	1	**9**	1	0	0
Left back	**9**	0	0	0	2	0	0
Right back	0	**7**	0	0	0	2	0
Left belly	**10**	1	2	0	0	1	0
Right belly	2	**13**	0	2	0	0	1

these lymph nodes, i.e. the front legs, and clones introduced into the rear legs often showed up in the POP nodes. However, many of the barcoded clones inoculated into the rear legs were also detected in FEM nodes, where they complemented the array of expected clones that had been intradermally injected into belly and back of the animals.

3.7. Interplay between lymphatic and gastrointestinal system

Frequently, barcoded clones that had been orally delivered to the animal were also found in peripheral lymph nodes, indicating that *Salmonella* readily crossed from the bovine gut environment into the MES and from there traversed into the lymphatic system of the animal. Approximately 27% of all clones detected in the PLNs of all 12 animals originated from an orally delivered *Salmonella* pool (Table 5). In the ten animals which had been sacrificed seven or eight days post inoculation (steers S3 – S8, MA1, MA2, MB1 and MB2), 50% of all peripheral lymph nodes (30 / 60) had been colonized with strains that had been delivered orally, and the overall contribution of those oral clones to the PLN population diversity was more than 31%. Very often (in 22 of these 30 cases), the colonized PLNs also contained one or more barcoded *Salmonella* isolate that had been introduced intradermally into the animal. However, in animals S1 and S2, we did not detect any oral isolates in the assayed PLNs.

Table 5 also shows that the mesenteric lymph nodes (MES) of the steers, which had been reliably colonized in all cases by multiple clones, presented almost exclusively clones that had been given orally to the steer. While transmission of clones from the gastrointestinal environment into the lymphatic system occurred quite frequently, colonization of the MES by intradermally delivered clones happened very rarely.

An enumeration of detected clone occurrences in all gastrointestinal loci sampled from all 12 animals (i.e. fecal samples, cecal contents and spiral colon contents) revealed that the vast majority of detected clones in that environment (93.8%) stemmed from oral pools. Transmission of intradermally delivered clones into the gastrointestinal environment was therefore rarer than the entry of orally delivered clones into the lymphatic system. Most of the few intradermally delivered clones that reached the gastrointestinal environment originated from belly (36%) or back (43%) intradermal delivery sites. No clones were observed in animals S1 and S2 that traversed into the gastrointestinal environment after intradermal delivery.

3.8. Extra-intestinal proliferation of Salmonella during or after transit to the PLN

The number of colony forming units obtained from the different lymph nodes in the different experiments did not provide conclusive evidence of bacterial proliferation over time in the node environment. In inoculation *scheme S*, steers that were sacrificed three days after inoculation harbored roughly the same number of bacteria in the lymph nodes compared with the steers that had been sacrificed after seven days (median numbers of bacteria per organ for all POP, FEM and SCAP were 42 for the three-day exposure, and 39 for the seven-day exposure, Table 3). In fifteen cases, the PLNs of the steers that had been sacrificed after seven days remained clear of barcoded *Salmonella*. In inoculation *scheme M*, a median count of 128 bacteria per node was found in the four animals that were inoculated with multiple deliveries of intradermal pools and harvested after seven days.

In order to investigate whether any proliferation of intradermally delivered isolates had occurred on their path to their expected draining peripheral lymph node in the animals, we obtained numerical ratios between the different barcoded clones of the same *S. enterica* strain colonizing the same expected lymph node, measured by the frequency of relevant barcode reads. Numbers were not compared between different strains, to exclude clone-specific genetic differences that might cause differential loss or proliferation in the animal. If ratios of genetically identical colonizing strains resembled the ratios observed in intradermal input pools, replication was not provable by this strategy. However, altered ratios would indicate occurrence of some replication on the way from the intradermal delivery site to the lymph node, and/or within the node itself. For barcoded clones from the same strain that occur in sufficient numbers in the organ, any observable differences in ratios cannot be caused by differential death because they are genetically identical, and must instead be caused by the difference in time when individual cells of different barcoded clones pass a severe bottleneck into a biological niche that then allows replication. Table 6 summarizes the seven cases where such altered ratios were observed among two or more barcoded clones of the same strain in a specific peripheral lymph node, when CFUs were higher than 250 per PLN. In these seven cases, some replication must have occurred.

Table 6
Proliferation of intradermally delivered *S. enterica* clones within cattle. The table illustrates observed ratio changes of multiple clones of the same *S. enterica* strain from intradermal inoculations detected with > 250 CFUs in bovine peripheral lymph nodes. Input ratios were calculated using median counts obtained from at least three input enumerations. Only clones with sequencing counts > 200 are shown. SMO, *S. enterica* serovar Montevideo; SAN, *S. enterica* sv Anatum; FEM, femoral lymph node; L, left; R, right.

Pool	Site	Strain	Clone IDs	Ratio, Input	Ratio, Output	Node	Cow#	CFU per Organ
TE4	Right belly	SMO-3	B51 : B50	3.4 : 1	12.9 : 1	FEMR	S2	423
A3R	Right rear leg	SMO-1	A25 : A26 : A27	3.1 : 3.2 : 1	0.6 : 0.6 : 1	FEMR	MA1	286
A3L / A4L	Left rear leg / belly	SMO-1	A22 : A23 : A24	3.0 : 1.2 : 1	32.4 : 0.6 : 1	FEML	MA2	741
A3R	Right rear leg	SMO-1	A25 : A27	3.1 : 1	11.2 : 1	FEMR	MA2	960
B3L	Left rear leg	SMO-1	B58 : B59 : B60	2.7 : 1.0 : 1	0.1 : 0.9 : 1	FEML	MB1	417
B5R	Right back	SAN-4	B72 : B73 : B74	1.4 : 0.9 : 1	4.1 : 0.2 : 1	FEMR	MB1	1520
B4R	Right belly	SMO-1	B66 : B67 : B68	2.6 : 1.4 : 1	32.2 : 1 : 1	FEMR	MB2	512

4. Discussion

Using a combination of an intradermal inoculation technique (Edrington et al., 2013b) and the STAMP technique based on the WITS molecular barcoding technology (Abel et al., 2015; Grant et al., 2008), we were able to delineate the hitherto unstudied dynamics of *Salmonella* colonization of steers after both oral and intradermal inoculation. Using these routes of delivery, bacterial colonization was readily established in cattle gastrointestinal tracts and peripheral lymph nodes.

For our studies, we chose to introduce DNA barcodes into the genome of *Salmonella* strains that had previously been harvested from cattle peripheral lymph nodes, and therefore may have been adapted to this ecological niche. In addition, we decided to utilize isolates covering various serovars, to be able to investigate possibly existing serovar-specific differences in bacterial transmission or colonization rates. We included strains representing the three most common *Salmonella* serovars found in US cattle feedlots (Anatum, Montevideo and Kentucky; http://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_is_Salm.pdf).

In our experiments, bacterial founder population sizes for gut and lymph node colonizations were determined to be in the hundreds and tens, respectively. A longer time prior to sacrifice might result in more bacterial founder colonies to establish colonization in the PLNs. Indeed, we noted that orally delivered bacterial isolates had not reached peripheral lymph nodes after three days (steers S1 and S2), but had successfully colonized some PLNs after seven or more days. However, previous observations suggest an eventual loss of intra-node bacteria after about 28 days (Edrington et al., 2016). While the total bacterial numbers in the peripheral lymph nodes in our experiments remained low, we found evidence of bacterial replication of intradermally delivered *Salmonella*. This replication must have occurred either after intradermal delivery, prior to arrival at the node and/or after arrival, intra-node. We were unable to distinguish unambiguously between these two possibilities. Further experiments will be needed to establish whether the bacteria replicate inside the PLNs, and at what frequency.

Our investigation of *Salmonella* transmission paths to the cattle peripheral lymph nodes after intradermal delivery of the bacteria yielded both expected and unexpected results. In most cases, bacteria drained from intradermal injection sites into the expected peripheral lymph node. However, subiliac / prefemoral nodes also often harbored bacteria that had been delivered to the rear legs. Since intradermal injections had been carefully applied to the mid- to lower limb area, subiliac drainage vessels may reach lower into the legs than anticipated in Holstein steers.

PLNs often harbored orally delivered bacterial barcoded clones, underlining the capability of bacterial populations to traverse the barriers from the gastrointestinal tract to the lymphatic system. However, this movement of bacterial communities between the two compartments may be mostly unidirectional - only a few intradermally delivered isolates were present in the animals' gut environment. It is possible that routes of cross-contamination by animal behaviors (licking, scratching) could play a role in the observed gastrointestinal presence of a few intradermal isolates. Further experimentation is needed to clarify whether clones do indeed traverse from the lymphatic system to the gastrointestinal environment while inside the animal.

The mesenteric lymph node exhibited bacterial loads and strain varieties very similar to the ones observed in the gastrointestinal tracts, suggesting considerable transmission of bacteria from the GI tract into the gut-associated lymphoid tissue. However, bacterial transfer to systemic organs was shown to be independent from bacterial transfer to MES, and vaccination strategies in mice prevented *Salmonella* uptake by the systemic organs while showing no effect on the bacterial uptake by the MES (Lim et al., 2014). The necessity to cross the barrier between these two systems for systemic disease may therefore represent an excellent target for vaccines.

Since PLNs are located deeply buried between muscles (Brashears and Chaves, 2017) and are therefore protected from post-harvest interventions, prevention of cattle lymph node colonization is of high importance. The low complexity of the lymph node bacterial founder population in our experiments, and the overall low total bacterial numbers obtained from the steers' PLNs, suggests that improved pre-harvest strategies may be able to substantially reduce or eliminate lymph node colonization. Currently applied pre-harvest strategies, such as direct-fed anti-microbials, vaccinations, bacteriophages (Loneragan and Brashears, 2005; Sargeant et al., 2007) and the controversial use of antibiotics on livestock (Helke et al., 2017) display some, but not total, effectiveness.

The design of an alternative successful intervention may be challenging given the reported failure to detect efficacy following administration of a *Salmonella* subunit vaccine (Cernicchiaro et al., 2016). The variable LPS that defines the different serovars of *Salmonella* may make broadly effective vaccines hard to achieve. Unfortunately, cattle can harbor a wide variety of *Salmonella* serovars (Gragg et al., 2013b; Kunze et al., 2008). Nevertheless, the fact that a *Salmonella* vaccine based on components of *Salmonella* Newport has some protective effect (Hermesch et al., 2008; Loneragan et al., 2012) suggests the concept of vaccination-by-preinoculation with a debilitated *S. enterica* isolate to be valid. In this respect, our data in inoculation *scheme S* suggest that natural pre-colonization with wild type Montevideo isolates may have prevented a successful crossing from the gastrointestinal environment to the lymphatic system by barcoded clones from the same serovar. However, these preliminary observations should be substantiated in more targeted experiments designed to study such a partial effect of pre-exposure to live bacteria. Notably, measurement of such an partial effect had been impossible until the advent of WITS and STAMP technologies (Abel et al., 2015). Further studies may elucidate the feasibility and possible partial protection of various vaccination approaches, by measuring the effect of these strategies on the founder population size in steer PLNs and organs, over longer periods of time.

Our experiments may be a model for future studies on partially protective vaccination in multi-route infection systems. In a typical efficacy study with a large challenge dose, a partially protective vaccine could reduce but not eliminate the bacterial load. This reduction would not be evident without characterization of the complexity of the pathogen's founder cells that are causing the colonization. Without such knowledge, partially protective candidate vaccines would be rejected in these studies as not efficacious, whereas in the field, where organisms are often exposed to small concentrations of pathogens, the vaccine could successfully lower the overall level of colonization in the population. The use of multiple barcoded challenge strains could expose any effect of the candidate vaccine on pathogen bottleneck size or persistence. Indeed, vaccines that have been rejected in other studies may be reinvestigated with this tool to determine if they have useful partially protective properties. Such partially protective vaccines are of particular interest for pathogens where 100% effective vaccines are difficult to develop, such as *Salmonella*.

In future experiments, our established library of *Salmonella* WITS clones can be applied to expose and investigate inter-animal bacterial transmission routes in feedlot cattle herds, a process that is of high importance when devising future strategies of pathogen colonization prevention in cattle.

5. Conclusion

We employed the STAMP strategy, where amplification of introduced barcoded regions was used to investigate the spatiotemporal dynamics of *Salmonella* colonization inside feedlot cattle. We improved the protocol to allow analysis to proceed on partially purified DNA in a single tube. Many different complex pools of barcoded strains, delivered to and recovered from different locations of the same animal, were rapidly analyzed in a small fraction of a sequencing lane. We applied this protocol to uncover the hitherto unknown migration patterns of

Salmonella in steers. Using this strategy, we determined the scale of the founder population sizes for bacterial colonies in different steer environments, with a minimal number of animals. Encouragingly, the founder population numbers for peripheral lymph node colonizations were low and extensive replication rare. Improvements in pre-harvest interventions may therefore be able to clear or prevent such infections in the future. The tools we present here will be immensely useful to assess pre-harvest interventions directed at prevention of contamination of meat products with *Salmonella* and may aid future studies on partially protective vaccination in cattle and other livestock.

Acknowledgements

This work was supported by the Foundation for Meat and Poultry Research and Education and by the National Cattlemen's Beef Association to Michael McClelland and Tom Edrington, and by USDA grant 2017-67015-26085 to Helene Andrews-Polymenis, on which Michael McClelland is a co-PI. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. We thank Helene Andrews-Polymenis for her invaluable comments on the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.05.007.

References

Abel, S., Abel zur Wiesch, P., Chang, H.H., Davis, B.M., Lipsitch, M., Waldor, M.K., 2015. Sequence tag-based analysis of microbial population dynamics. Nat. Methods 12, 223–226.

Arthur, T.M., Brichta-Harhay, D.M., Bosilevac, J.M., Guerini, M.N., Kalchayanand, N., Wells, J.E., Shackelford, S.D., Wheeler, T.L., Koohmaraie, M., 2008. Prevalence and characterization of Salmonella in bovine lymph nodes potentially destined for use in ground beef. J. Food Prot. 71, 1685–1688.

Brashears, M.M., Chaves, B.D., 2017. The diversity of beef safety: a global reason to strengthen our current systems. Meat Sci. 132, 59–71.

Brichta-Harhay, D.M., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Schmidt, J.W., Wang, R., Shackelford, S.D., Loneragan, G.H., Wheeler, T.L., 2012. Microbiological analysis of bovine lymph nodes for the detection of Salmonella enterica. J. Food Prot. 75, 854–858.

Canals, R., Xia, X.Q., Fronick, C., Clifton, S.W., Ahmer, B.M., Andrews-Polymenis, H.L., Porwollik, S., McClelland, M., 2012. High-throughput comparison of gene fitness among related bacteria. BMC Genomics 13, 212.

Cernicchiaro, N., Ives, S.E., Edrington, T.S., Nagaraja, T.G., Renter, D.G., 2016. Efficacy of a Salmonella siderophore receptor protein vaccine on fecal shedding and lymph node carriage of Salmonella in commercial feedlot cattle. Foodborne Pathog. Dis. 13, 517–525.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. 97, 6640–6645.

de Moraes, M.H., Desai, P., Porwollik, S., Canals, R., Perez, D.R., Chu, W., McClelland, M., Teplitski, M., 2017. Salmonella persistence in tomatoes requires a distinct set of metabolic functions identified by transposon insertion sequencing. Appl. Environ. Microbiol. 83 (5), e03028–16.

Edrington, T.S., Loneragan, G.H., Genovese, K.J., Hanson, D.L., Nisbet, D.J., 2016. Salmonella persistence within the peripheral lymph nodes of cattle following experimental inoculation. J. Food Prot. 79, 1032–1035.

Edrington, T.S., Loneragan, G.H., Hill, J., Genovese, K.J., Brichta-Harhay, D.M., Farrow, R.L., Krueger, N.A., Callaway, T.R., Anderson, R.C., Nisbet, D.J., 2013a. Development of challenge models to evaluate the efficacy of a vaccine to reduce carriage of Salmonella in peripheral lymph nodes of cattle. J. Food Prot. 76, 1259–1263.

Edrington, T.S., Loneragan, G.H., Hill, J., Genovese, K.J., He, H., Callaway, T.R., Anderson, R.C., Brichta-Harhay, D.M., Nisbet, D.J., 2013b. Development of a transdermal Salmonella challenge model in calves. J. Food Prot. 76, 1255–1258.

Gragg, S.E., Loneragan, G.H., Brashears, M.M., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Wang, R., Schmidt, J.W., Brooks, J.C., Shackelford, S.D., Wheeler, T.L., Brown, T.R., Edrington, T.S., Brichta-Harhay, D.M., 2013a. Cross-sectional study examining Salmonella enterica carriage in subiliac lymph nodes of cull and feedlot cattle at harvest. Foodborne Pathog. Dis. 10, 368–374.

Gragg, S.E., Loneragan, G.H., Nightingale, K.K., Brichta-Harhay, D.M., Ruiz, H., Elder, J.R., Garcia, L.G., Miller, M.F., Echeverry, A., Ramirez Porras, R.G., Brashears, M.M., 2013b. Substantial within-animal diversity of Salmonella isolates from lymph nodes, feces, and hides of cattle at slaughter. Appl. Environ. Microbiol. 79, 4744–4750.

Grant, A.J., Restif, O., McKinley, T.J., Sheppard, M., Maskell, D.J., Mastroeni, P., 2008. Modelling within-host spatiotemporal dynamics of invasive bacterial disease. PLoS Biol. 6, e74.

Haneklaus, A.N., Harris, K.B., Griffin, D.B., Edrington, T.S., Lucia, L.M., Savell, J.W., 2012. Salmonella prevalence in bovine lymph nodes differs among feedyards. J. Food Prot. 75, 1131–1133.

Hao, L.Y., Willis, D.K., Andrews-Polymenis, H., McClelland, M., Barak, J.D., 2012. Requirement of siderophore biosynthesis for plant colonization by Salmonella enterica. Appl. Environ. Microbiol. 78, 4561–4570.

Helke, K.L., McCrackin, M.A., Galloway, A.M., Poole, A.Z., Salgado, C.D., Marriott, B.P., 2017. Effects of antimicrobial use in agricultural animals on drug-resistant foodborne salmonellosis in humans: a systematic literature review. Crit. Rev. Food Sci. Nutr. 57, 472–488.

Hermesch, D.R., Thomson, D.U., Loneragan, G.H., Renter, D.R., White, B.J., 2008. Effects of a commercially available vaccine against Salmonella enterica serotype Newport on milk production, somatic cell count, and shedding of Salmonella organisms in female dairy cattle with no clinical signs of salmonellosis. Am. J. Vet. Res. 69, 1229–1234.

Kaiser, P., Slack, E., Grant, A.J., Hardt, W.D., Regoes, R.R., 2013. Lymph node colonization dynamics after oral Salmonella Typhimurium infection in mice. PLoS Pathog. 9, e1003532.

Kaniuk, N.A., Canadien, V., Bagshaw, R.D., Bakowski, M., Braun, V., Landekic, M., Mitra, S., Huang, J., Heo, W.D., Meyer, T., Pelletier, L., Andrews-Polymenis, H., McClelland, M., Pawson, T., Grinstein, S., Brumell, J.H., 2011. Salmonella exploits Arl8B-directed kinesin activity to promote endosome tubulation and cell-to-cell transfer. Cell. Microbiol. 13, 1812–1823.

Koohmaraie, M., Scanga, J.A., De La Zerda, M.J., Koohmaraie, B., Tapay, L., Beskhlebnaya, V., Mai, T., Greeson, K., Samadpour, M., 2012. Tracking the sources of salmonella in ground beef produced from nonfed cattle. J. Food Prot. 75, 1464–1468.

Kunze, D.J., Loneragan, G.H., Platt, T.M., Miller, M.F., Besser, T.E., Koohmaraie, M., Stephens, T., Brashears, M.M., 2008. Salmonella enterica burden in harvest-ready cattle populations from the southern high plains of the United States. Appl. Environ. Microbiol. 74, 345–351.

Lim, C.H., Voedisch, S., Wahl, B., Rouf, S.F., Geffers, R., Rhen, M., Pabst, O., 2014. Independent bottlenecks characterize colonization of systemic compartments and gut lymphoid tissue by salmonella. PLoS Pathog. 10, e1004270.

Loneragan, G.H., Brashears, M.M., 2005. Pre-harvest interventions to reduce carriage of E. coli O157 by harvest-ready feedlot cattle. Meat Sci. 71, 72–78.

Loneragan, G.H., Thomson, D.U., McCarthy, R.M., Webb, H.E., Daniels, A.E., Edrington, T.S., Nisbet, D.J., Trojan, S.J., Rankin, S.C., Brashears, M.M., 2012. Salmonella diversity and burden in cows on and culled from dairy farms in the Texas High Plains. Foodborne Pathog. Dis. 9, 549–555.

Narvaez-Bravo, C., Miller, M.F., Jackson, T., Jackson, S., Rodas-Gonzalez, A., Pond, K., Echeverry, A., Brashears, M.M., 2013. Salmonella and Escherichia coli O157:H7 prevalence in cattle and on carcasses in a vertically integrated feedlot and harvest plant in Mexico. J. Food Prot. 76, 786–795.

Olafson, P.U., Brown, T.R., Lohmeyer, K.H., Harvey, R.B., Nisbet, D.J., Loneragan, G.H., Edrington, T.S., 2016. Assessing transmission of Salmonella to bovine peripheral lymph nodes upon horn fly feeding. J. Food Prot. 79, 1135–1142.

Reynolds, M.M., Bogomolnaya, L., Guo, J., Aldrich, L., Bokhari, D., Santiviago, C.A., McClelland, M., Andrews-Polymenis, H., 2011. Abrogation of the twin arginine transport system in Salmonella enterica serovar Typhimurium leads to colonization defects during infection. PloS One 6, e15800.

Santiviago, C.A., Blondel, C.J., Quezada, C.P., Silva, C.A., Tobar, P.M., Porwollik, S., McClelland, M., Andrews-Polymenis, H.L., Toro, C.S., Zaldivar, M., Contreras, I., 2010. Spontaneous excision of the Salmonella enterica serovar enteritidis-specific defective prophage-like element phiSE14. J. Bacteriol. 192, 2246–2254.

Santiviago, C.A., Reynolds, M.M., Porwollik, S., Choi, S.H., Long, F., Andrews-Polymenis, H.L., McClelland, M., 2009. Analysis of pools of targeted Salmonella deletion mutants identifies novel genes affecting fitness during competitive infection in mice. PLoS Pathog. 5, e1000477.

Sargeant, J.M., Amezcua, M.R., Rajic, A., Waddell, L., 2007. Pre-harvest interventions to reduce the shedding of E. coli O157 in the faeces of weaned domestic ruminants: a systematic review. Zoonoses Public Health 54, 260–277.

Silva, C.A., Blondel, C.J., Quezada, C.P., Porwollik, S., Andrews-Polymenis, H.L., Toro, C.S., Zaldivar, M., Contreras, I., McClelland, M., Santiviago, C.A., 2012. Infection of mice by Salmonella enterica serovar Enteritidis involves additional genes that are absent in the genome of serovar Typhimurium. Infect. Immun. 80, 839–849.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., McClelland, M., Shi, Y., 2011. The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. J. Biol. Chem. 286, 5529–5539.

Webb, H.E., Brichta-Harhay, D.M., Brashears, M.M., Nightingale, K.K., Arthur, T.M., Bosilevac, J.M., Kalchayanand, N., Schmidt, J.W., Wang, R., Granier, S.A., Brown, T.R., Edrington, T.S., Shackelford, S.D., Wheeler, T.L., Loneragan, G.H., 2017. Salmonella in peripheral lymph nodes of healthy cattle at slaughter. Front. Microbiol. 8, 2214.

Yang, Y., Ricke, S.C., Tellez, G., Kwon, Y.M., 2017. Quantitative tracking of Salmonella Enteritidis transmission routes using barcode-tagged isogenic strains in chickens: proof-of-concept study. Front. Vet. Sci. 4, 15.